REMARKS

Entry of the foregoing amendment, and further favorable reconsideration of the subject application in light of the following remarks, pursuant to and consistent with 37 C.F.R. § 1.112, are respectfully requested.

By the present amendment, claims 1, 2, 4-8, and 19 have been amended to rephrase the recitation of the claimed invention. Claims 23 and 24 have been added to recite subject matter stricken from claim 4. Support for the amendments can be found throughout the disclosure as filed, particularly the claims as originally filed. No new matter has been added.

No prohibited new matter has been introduced by way of the above amendments. Applicants reserve the right to file a continuation or divisional application on any subject matter that may have been canceled by way of this amendment.

Objection To The Specification

The Examiner has indicated that the Specification is not in sequence compliance. The Examiner's reference to a sequence at page 20, line 30, suggests that the Examiner is considering the specification as published in the International Application. The Examiner's attention is directed to the Substitute Specification introduced by the Preliminary Amendment filed May 19, 2005.

By the present amendment, the Substitute Specification has been amended so that each sequence recited therein is accompanied by a reference to the sequence listing at an appropriate location. The brief description of Figure 1 has been amended to refer to the sequence listing.

The Specification has also been objected to for containing hyperlinks. According to Manual of Patent Examination Procedure § 608.01, text is potentially browser executable

hyperlink if it contains brackets "<>" and the code "http://." The Substitute Specification has been amended to delete an occurrence of "http://" thereby rendering the URL associated therewith as merely simple text.

Restriction Requirement

Claims 6, 11-15 and 19-22 have been withdrawn from consideration by the Examiner pursuant to a restriction requirement, which has been made final. The Examiner has alleged that the claims recite no special technical feature with respect to the prior art represented by Warren et al. Applicants have traversed the restriction requirement and maintain that the requirement is improper as each of the withdrawn claims shares unity of invention with the claims under consideration.

Applicants have pointed out that each of the claims as currently presented contain a special technical feature represented by the use of primers associated with SEQ ID NOS: 76 and 77, which amplify regions of the gyrB and parE genes, and probes specific for the gyrB and parE genes. Warren et al. specifically provides polynucleotides encoding gyrA and discloses only the use of probes and primers associated therewith. Warren et al. does not teach or suggest SEQ ID NOS: 76 or 77, or probes and primers associated with gyrB and/or parE.

The Examiner's reason for maintaining the rejection, alleged for the first time in the present Office Action, is that Warren teaches primers SEQ ID NOS: 3 and 4comprising the sequences "ATA" and "CCGC" near the 3' ends, and that these fragments of Warren's primer sequences are also found near the 3' ends of SEQ ID NOS:76 and 77 recited in the present claims. The Examiner has asserted that "the 3' end of a primer is the functional end

of a primer." By this, the Examiner is understood to suggest that as a general rule, sequence at the 3' end of a primer can have a greater impact on primer function than at the 5' end.

Even if Warren's primers comprise fragments of SEQ ID NOS:76 and 77, Warren et al. has not disclosed any primer comprising a sequence of SEQ ID NO:76 or 77 or a "*functional* fragment thereof." Applicants respectfully submit that a person of ordinary skill in the art would not reasonably construe "functional fragment" to mean the portion of a primer sequence without which a primer will not function, nor has any evidence been adduced that the identified fragmentary sequences are required for function of the primers recited in the present claims. A "functional fragment" can only be understood to mean at least a fragment sufficient to function as a primer for the step in the method in which it is recited.

The functional design requirements of primers are well known to persons of ordinary skill in the art. *See, e.g.*, Sharrocks, *The Design of Primers for PCR* in "PCR Technology: Current Innovations" (CRC Press, 1994; Griffin and Griffin, eds.); attached as Exhibit A. It was understood that if the 5' end of a primer incorporates a tag or adapter not found in the target sequence, 15-18 contiguous gene specific nucleotides are desirable on the 3' end of the primer. *Id.* at 8, first full paragraph; see also Hogan et al. at col. 10, lines 31-34 (teaching that for primer sequences of 15-20 nucleotides, 75-100% homology with a target sequence is required.)

Applicants respectfully submit that a person of ordinary skill in the art would understand that a trinucleotide sequence or a 4-mer cannot reasonably constitute a "functional fragment" of a primer in the context of the present claim read in light of the specification. Read in the context of the claim as a whole in light of the specification, as it must be, one of skill in the art would understand that a "functional fragment" of a recited primer sequence

must comprise at least enough of the recited primer sequence to function as a primer for amplification of the sequences which the recited primer sequence as a whole amplifies. More particularly, a person of ordinary skill reading the claim as a whole in light of the specification would understand that the recited "functional fragment" of a primer sequence must be functional in the step of "amplifying DNA isolated from [a] clinical sample using a mixture of DNA primers comprising sequences which hybridize with conserved regions of genes encoding topoisomerases of infectious bacterial species."

Warren et al. has not taught or suggested any primer that comprises either SEQ ID NO:76 or 77 or a functional fragment thereof. Therefore, the claims of the application do recite at least one special technical feature in view of the prior art, and the restriction requirement is improper. Accordingly, Applicants will file a petition under 37 C.F.R. §§ 1.144 and 1.181.

Rejections under 35 U.S.C. § 112

Claims 1-5, 7-10 and 13 have been rejected under 35 U.S.C. § 112 as allegedly indefinite for lacking a positive action step relating back to the preamble. This rejection is traversed. The Examiner has cited no authority either in the M.P.E.P. or case law indicating that a positive action step relating back to the preamble is required in a method claim. Applicants respectfully submit that a person of ordinary skill in the art reading the claims as amended in light of the specification would clearly understand that the recited steps are sufficient to accomplish the purpose of the method stated in the preamble.

Claims 1-5, 7-10 and 13 have been rejected under 35 U.S.C. § 112 as allegedly indefinite for reciting a "desired combination." The rejection is traversed. Without acceding to the allegation, the claims have been rephrased so as not to use the term "desired."

Claims 1-5, 7-10 and 13 have been rejected under 35 U.S.C. § 112 as allegedly indefinite for reciting "said sequences." The rejection is traversed. Without acceding to the allegation, the claims have been rephrased. Applicants respectfully submit that the claims as amended clearly describe the claimed invention.

Claims 1-5, 7-10 and 13 have been rejected under 35 U.S.C. § 112 as allegedly indefinite for reciting "possible." The rejection is traversed. Without acceding to the allegation, the claims have been rephrased to strike the word "possible." Applicants respectfully submit that a person of ordinary skill in the art would understand that probes is a term used for oligonucleotides designed for the detection of hybridization and primers is a term used for oligonucleotides designed for amplification so that the hybridization complex being detected would be understood to be between the probe and the hypervariable-region as recited in the penultimate step.

Claim 4 has been rejected under 35 U.S.C. § 112 as allegedly indefinite for reciting nested ranges. Claim 4 has been amended to strike the nested ranges, which are now recited in claims 23 and 24.

Claim 8 has been rejected under 35 U.S.C. § 112 as allegedly indefinite for reciting "the DNA isolated." Claim 8 has been amended for consistency with claim 1

Applicants respectfully submit that the claims as amended distinctly describe and particularly point out the claimed invention.

Rejections under 35 U.S.C. § 102

Claims 1-2 and 4 have been rejected under 35 U.S.C. § 102 as allegedly anticipated by U.S. Patent Number 5,645,994 ("Huang"). The rejection is traversed.

The rejection is premised upon a construction of the claim that is impermissibly inconsistent with any interpretation that a person of ordinary skill in the art would reasonably reach. Although the PTO must give claims their broadest reasonable interpretation, this interpretation must be consistent with the one that those skilled in the art would reach. *In re Cortright*, 165 F.3d 1353, 1358, 49 USPQ2d 1464 (Fed. Cir. 1999). In this "the PTO applies to the verbiage of the proposed claims the broadest reasonable meaning of the words in their ordinary usage as they would be understood by one of ordinary skill in the art . . . ." *In re Morris*, 127 F.3d 1048, 1054, 44 USPQ2d 1023, 1027 (Fed.Cir. 1997); In re Bond , 910 F.2d 831, 833, 15 USPQ2d 1566, 1567 (Fed.Cir. 1990) ("It is axiomatic that, in proceedings before the PTO, claims in an application are to be given their broadest reasonable interpretation consistent with the specification, . . . and that claim language should be read in light of the specification *as it would be interpreted by one of ordinary skill in the art.*") (emphasis added); see also M.P.E.P. Section 2111.01 ("[T]he words of a claim . . . must be read as they would be interpreted by those of ordinary skill in the art.").

The Examiner has stated that "the specification does not specifically define a functional fragment, thus a functional fragment is broadly interpreted to be at least a single nucleotide." Applicants respectfully submit that a person of ordinary skill in the art using the plain meaning of the term "functional fragment" would not consider that the term embraced a single nucleotide. A "*functional* fragment" can only be understood to mean at least a fragment sufficient to be *functional* as a primer for the step in the method in which it is recited.

The functional design requirements of primers are well known to persons of ordinary skill in the art. *See, e.g.*, Sharrocks, *The Design of Primers for PCR* in "PCR Technology: Current Innovations" (CRC Press, 1994; Griffin and Griffin, eds.); attached as Exhibit A. It

was understood that if the 5' end of a primer incorporates a tag or adapter not found in the target sequence, 15-18 contiguous gene specific nucleotides are desirable on the 3' end of the primer. *Id.* at 8, first full paragraph; see also Hogan et al. at col. 10, lines 31-34 (teaching that for primer sequences of 15-20 nucleotides, 75-100% homology with a target sequence is required.)

Read in the context of the claim as a whole in light of the specification, as it must be, one of skill in the art would understand that a "functional fragment" of a recited primer sequence must comprise at least enough of the recited primer sequence to function as a primer for amplification of the sequences which the recited primer sequence as a whole amplifies. More particularly, a person of ordinary skill reading the claim as a whole in light of the specification would understand that the recited "functional fragment" of a primer sequence must be functional in the step of "amplifying DNA isolated from [a] clinical sample using a mixture of DNA primers comprising sequences which hybridize with conserved regions of genes encoding topoisomerases of infectious bacterial species."

The Examiner has alleged that Huang et al. in column 6, lines 28-65 teach a method of designing universal primers that amplify *parE* and *gyrB* and that these primers would identify the sequences that primers 76 and 77 would identify. This is not correct. In the cited text, Huang describes the principle behind designing universal primers in general, without reference to any sequence. The present claims do not recite universal primers in general, neither do the present claims recite universal primers that amplify and identify topoisomerases in general. The present claims relate to a diagnostic method for detecting infectious bacterial species in a sample, which method makes use of a specific (species independent and thus universal) primer pair cocktail comprising the sequences of SEQ ID NOS: 76 and 77 for amplifying that specific stretch of DNA of the topomerase encoding gene

and of species-specific probes that hybridize to specific sites contained in that amplified sequence.

Huang et al. describe primers for gyrB/gyrE. However, the Examiner has presented no comparison showing that the Huang primers comprise SEQ ID NOS:76 and 77 or functional fragments thereof using a reasonable interpretation of "functional fragment." Neither has Huang et al. even suggested making primers comprise SEQ ID NOS:76 and 77 or functional fragments thereof. The reverse primer according to Huang et al. is homologous to a sequence much closer to the forward primer, when compared to the primers of the present invention. Thus the target sequence amplified by the primer pair of Huang et al. is much shorter than the sequence amplified by the primer pair 76 and 77. The missing part of the target sequence provides sequence important for developing species specific probes.

"A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 U.S.P.Q.2d 1051, 1053 (Fed. Cir. 1987). The elements must be arranged as required by the claim. *In re Bond*, 910 F.2d 831, 15 U.S.P.Q.2d 1566 (Fed. Cir. 1990).

Huang et al. has not disclosed a method comprising all the required elements of the present claims and cannot anticipate the claims.

Rejections under 35 U.S.C. § 103

Claims 3, 7, 8, 10 and 13 have been rejected under 35 U.S.C. § 103 as allegedly unpatentable over Huang in view of U.S. Patent Publication 2004/0048281 ("Voelker"). Claim 9 has been rejected under 35 U.S.C. § 103 as allegedly unpatentable over Huang in

view of U.S. Patent Publication 2004/0048281 ("Voelker") and Southern et al. *Nature Genetics* 21 (Supplement):5-9, 1999. These rejections are traversed.

The prior art fails to establish a proper prima facie case of obviousness. To establish a prima facie case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. M.P.E.P. § 2143.

The deficiencies of Huang are discussed above when compared to the claims using an interpretation that a person of ordinary skill in the art would reasonably reach. Voelker has been alleged to teach the amplification of gram-positive bacteria, S. aureus gyrB and parE and the use of degenerate primers for the identification of quinilone resistance determining regions across phylogenetic ranges of prokaryotes.

However, Voelker et al. do not teach or suggest the present invention. Voelker et at. do not describe species identification, but the identification quinolone resistance areas in gyrA-, gyrB-, parE- and parC genes of different bacteria.

Voelker teaches the amplification of a gene area which is different from the one amplified by the primer pair according to the presently claimed invention. It is generally known, that the quinolone resistance is due to mutations in the gyrA gene, but some corresponding areas in the gyrB gene are also known to contain mutation sites. By contrast. the gene area amplified in the present invention is chosen so as to not contain the most common mutation sites, because such sites would lead to polymorphisms making species specific identification difficult. Southern is directed to use of glass as a solid support in

hybridization, but does not cure the deficiencies of Huang and Voelker with respect to claim 9.

Claim 5 has been rejected under 35 U.S.C. § 103 as allegedly unpatentable over Huang in view of U.S. Patent Number 5,541,308 (Hogan) and Hopewell et al. (J. Bacteriology, 172:3481-41, 1990). This rejection is traversed.

The deficiencies of Huang are discussed above when compared to the claims using an interpretation that a person of ordinary skill in the art would reasonably reach. Hopewell and Hogan do not cure the deficiencies of Huang. Hopewell et at., describes mutation sites in the *E. coli* gyrB gene (Asp-426 and Lys-447), which correspond to nucleotides 1276-8 and 13339-41 (page 3483, column 1, line 10), whereas the gene area used in the presently claimed method is located within the 400 first nucleotides of the gyrB gene. Hogan teaches general principles of functional probe design that demonstrate that the Examiner's interpretation of "functional fragment" cannot be one that a person of ordinary skill in the art would reasonably reach.

Hopewell et al. teach the use of gyrB gene fragments from B. subti!is for identifying the gyrB gene of S. aureus. Hopewell is alleged to teach *S. aureus* sequences that comprise SEQ ID NO. 24, and using the Hopewell sequence or a fragment thereof one would be motivated to use that sequence in the present invention. It must be noted that the Examiner is not correct in stating that methicillin resistance is due to mutations in the gyrase enzymes. As a matter of fact this resistance is due to the SSCmec cassette, whereas the resistance afforded by gyrase mutations is expressed against quinolone antibiotics.

Nothing in Hopewell indicates that SEQ ID NO. 24 should be selected in order to design a *S. aureus* specific probe that would be useful in a diagnostic method according to the present invention.

CONCLUSION

In view of the foregoing, further and favorable action in the form of a Notice of Allowance is believed to be next in order. Such action is earnestly solicited.

In the event that there are any questions relating to this application, it would be appreciated if the Examiner would telephone the undersigned concerning such questions so that prosecution of this application may be expedited.

The Director is hereby authorized to charge any appropriate fees that may be required by this paper, and to credit any overpayment, to Deposit Account No. 02-4800.

Respectfully submitted,

BUCHANAN INGERSOLL & ROONEY PC

Date: May 29, 2007

By: _____
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PCR TECHNOLOGY
Current Innovations

Edited by

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Annette M. Griffin

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Chapter 2

THE DESIGN OF PRIMERS FOR PCR

Andrew D. Sharrocks

TABLE OF CONTENTS

I. INTRODUCTION

The development of the polymerase chain reaction (PCR) has revolutionized the field of molecular biology. PCR has been used for a plethora of applications, many of which are covered in this book. These applications involve both novel procedures (e.g., gene amplification from nanograms of genomic DNA[1,2]) and modifications of existing methods (e.g., site-directed mutagenesis[3,4]). Although many variables need to be optimized in the design of PCR-based procedures for each of these various applications, the most critical parameter in all cases is the correct designing of PCR primers. Indeed, the correct choice of PCR primers often dictates the success or failure of the PCR amplification. Careful design of primers can therefore save valuable research time, and in addition, can lead to significant savings in costs as the primers usually represent the most expensive component in a PCR.

One of the principal considerations in the design of a PCR protocol is to obtain unique, specific products as dictated by the selected primers. The first step in PCR primer design is to ensure this specificity. However, after specificity has been assured, further manipulation of the PCR primer design is possible. This allows the introduction of novel genetic information into the product. Such alterations range from single point mutations to the tagging of products with new coding sequences or regulatory elements. By careful and thoughtful primer design, specific products can be produced from a PCR with a multitude of possible engineered features.

Most of the rules for primer design are empirical with no guarantee of success. However, careful adherence to these rules will significantly increase the probability of a successful PCR. Computer programs are especially useful in the assessment of the basic parameters governing primer design. Even so, some primer pairs that fulfill all known criteria still fail to work for obscure reasons. This chapter provides a guide to reducing the possibility of an unsuccessful PCR but simultaneously demonstrates the flexibility that can be incorporated into primer design for product manipulation.

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II. GENERAL RULES FOR PRIMER DESIGN

Successful primer design and hence successful PCRs rely on the unique annealing of the two primers to the template with both high specificity and high efficiency. This ensures that only the desired product is synthesized. The problem of non-specific amplifications is intensified during the early rounds, when amplifications are performed on very small quantities of target DNA, which is often immersed in an excess of non-specific sequences. Correct annealing at this stage is imperative or errors will be compounded throughout the ensuing PCR.

Several parameters must be carefully considered in order to ensure correct annealing (Table 1). The first of these is to choose primers that have a sequence unique within the region to be amplified. The most important region to check is at the 3′ end of the primer as this is where synthesis of the PCR product begins. Such a procedure is tedious when executed manually but is handled easily by computer programs (see below). The second parameter to consider is the inclusion of a G/C residue at the 3′ end of the primer. This "G.C clamp" helps to ensure correct annealing at the 3′ end due to the strong hydrogen bonding utilized by G/C base pairs.

Primers should also be designed with no self-homology. Such self-homology can lead to partially double-stranded "snap back" structures that render the primer incapable of hybridizing to the template. A general rule of thumb is that no self-homology involving four contiguous base pairs should be present in the primer. A related parameter is that primers should show no homology to their antisense counterparts. Formation of partial hybrids between primer pairs can lead to the formation of "primer-dimers" in the ensuing PCR. Elimination of this artifact is essential as this by-product can easily swamp a PCR. Particular care should again be taken in removing complementarity between the 3′ ends of the two primers.

The primer base composition should also be closely monitored. In general a G/C content between 45 and 55% should be selected to direct specific binding yet allow efficient melting during the PCR. Efforts should also be made to keep the base composition close to that exhibited by the amplified region. In addition, the base distribution of the primers should be random, with polypurine and polypyrimidine tracts avoided. Nucleotide sequence repeats should also be avoided in primer design. The target sequence-specific part of primers should ideally be between 18 and 25 bases long. It is also important to have primer pairs with similar melting temperatures (T_m). This can be accurately calculated using the nearest-neighbor method with the formula: $T_m^{primer} = \Delta H/[\Delta S + R \ln (c/4)] - 273.15°C + 16.6 \log_{10} [K^+]$ where ΔH and ΔS are the enthalpy and entropy for helix formation, respectively, R is the molar gas constant, and c is the concentration of probe.[5] However, approximate T_ms can be calculated manually using the simpler formula $T_m = 2AT + 4GC$.[6] An equity in primer T_ms ensures simultaneous annealing of the primers. The calculated T_m can then be incorporated into the PCR protocol to optimize specific binding. This can be exploited when two sets of primer pairs with different matched T_ms are used in a single PCR to amplify different specific fragments.

An additional parameter that can be incorporated into primer design is to ensure that the T_m of the amplified region between the primers is low enough to ensure 100% melting at 92°C. This can be calculated using the formula $T_m = 81.5 + 16.6 (\log_{10}[K^+]) + 0.41 (\%G + C) - 675/\text{length}$.[7] This reduces to $T_m = 59.9 + 0.41 (\%G + C) - 675/\text{length}$ at standard PCR conditions containing 50 m*M* KCl. Inefficient melting will ultimately lead to a reduced yield from the PCR. Finally it is useful to design primers whose annealing sites are spaced between 100 and 600 bp. This distance allows efficient synthesis of product during the PCR.[8]

Adherence to the above parameters in designing primers for a PCR helps ensure specificity of the product. Such specificity is essential in applications where the non-target DNA is in great excess over the target DNA. Many of these PCR applications, covered in this volume, include procedures involving amplifications from genomic DNA, gene libraries, and whole cells.

Gene

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1. Unique oligonucle
2. G.C clamp at the
3. No self-compleme
4. No complementari
5. Random base distr
6. Primer length
7. Match primer T_ms
8. Distance and com

III. MODIFI

A. GENE AMPLIFICATIO

The use of PCR in gene a techniques. Both these applicat excess of non-specific DNA. I specific product amplification.

It is advisable to choose prin in the genome of the organism given genome is not yet known deposited in databases. Compu

In PCRs performed on cDN of primers that lie in different easily diagnosed. This is of extr siting of PCR primers in differ to information about differenti conventional cDNA cloning te

PCR is often used for clon sequence are gained from seq alternatively by deriving cons families or from related species on these peptide sequences an when designing such primers deduced sequence. This will h of product synthesis. The use successfully amplify the desir bring success, but rules gover emphasized that the PCR profi which will have low T_ms.

B. GENE MANIPULATIO

PCR not only allows specif manipulation of the cloned DN is the introduction of restrictio of the PCR product and the ir elements or additional coding

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during the PCR.[8]

or a PCR helps ensure specificity
where the non-target DNA is in
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TABLE 1.
General Rules for PCR Primer Design

Parameter	Optimum values
1. Unique oligonucleotide sequence	
2. G.C clamp at the 3′ end	1–2 G/C nucleotides
3. No self-complementarity	≤3 contiguous bases
4. No complementarity to antisense counterpart	≤3 contiguous bases
5. Random base distribution and composition	45–55% G/C content
6. Primer length	18–25 bases
7. Match primer T_ms	
8. Distance and composition of intraprimer sequence	100–600 bases apart

III. MODIFICATIONS TO PCR PRIMER DESIGN

A. GENE AMPLIFICATION

The use of PCR in gene amplification has applications in gene cloning and diagnostic techniques. Both these applications involve the amplification of specific sequences from a vast excess of non-specific DNA. In general the rules described above must be followed to give specific product amplification. However certain extra criteria must be considered (Table 2).

It is advisable to choose primer pairs that are unique not only within the target gene but also in the genome of the organism under investigation. As the entire nucleotide sequence of any given genome is not yet known, searches must obviously be limited to known gene sequences deposited in databases. Computer assistance is essential for this procedure.

In PCRs performed on cDNA templates, it is beneficial to design primer pairs consisting of primers that lie in different exons. In this way, contamination from genomic DNA can be easily diagnosed. This is of extreme importance if low transcript levels are to be detected. The siting of PCR primers in different exons has the additional benefit of being able to also lead to information about differential transcript splicing, a task that is extremely laborious using conventional cDNA cloning techniques.

PCR is often used for cloning genes that have largely unknown sequences. Clues to a sequence are gained from sequencing peptides derived from the purified gene product or alternatively by deriving consensus peptide sequences either from other members of gene families or from related species. Pairs of degenerate PCR primers can then be designed based on these peptide sequences and used in gene amplifications.[9,10] One important consideration when designing such primers is to site the 3′ end at the most complementary region of the deduced sequence. This will help ensure accurate primer annealing and the correct initiation of product synthesis. The use of multiple combinations of primer pairs is often necessary to successfully amplify the desired gene. Slight alterations in the position of the primers may bring success, but rules governing this type of analysis are largely empirical. It should be emphasized that the PCR profile must be altered to ensure annealing of degenerate primers, which will have low T_ms.

B. GENE MANIPULATION

PCR not only allows specific amplification of products but also allows the simultaneous manipulation of the cloned DNA sequence. One application that takes advantage of the latter is the introduction of restriction sites into the product for cloning.[2] Site-directed mutagenesis of the PCR product and the insertion of larger DNA fragments encoding either regulatory elements or additional coding sequences are also frequently used modifications.

Restriction enzyme recognition sequences can be engineered into PCR primers either individually or alternatively in groups on a 5′ extension known as the adaptor.[2] Commonly

TABLE 2.
Modifications of Primer Design for Specific Applications

Application	Parameters to be optimized
	Test uniqueness in genome
Gene amplification	
cDNA amplifications	Site primers in different exons
Degenerate primers	Site 3′ end in least ambiguous region
Gene manipulation	
Restriction site engineering	Locate recognition site >4 bases from 5′ end
Product tagging	Retain 15 — 18 specific bases at 3′ end
	Test adaptors for non-specific hybridization
"Two-step" site-directed mutagenesis	Site 5′ end of primer next to a T residue or in a codon wobble position
	Locate mismatches in the center of the primer
	Add 1.5 extra specific bases per mismatch

engineered restriction sites include those for NdeI and NcoI, which are used to allow the direct cloning of genes into expression vectors using either the natural or artificial ATG start codons. In addition PCR can be used to introduce restriction sites with concomitant silent mutations in the coding sequence. These sites can subsequently be used in domain swapping experiments. One important parameter to consider in designing these primers is that restriction enzyme cleavage is inefficient when its site is located toward the end of a DNA fragment. It is therefore advisable to position restriction sites at least 4 bp from the 5′ end of the primer. This will ensure efficient product cleavage by most restriction enzymes.[11]

The 5′ adaptors on primers can also be used to introduce larger stretches of additional sequence onto the products. Such sequences include promoter sequences (allowing direct *in vitro* transcription of PCR products[12,13]) and epitope tags (allowing antibody screening of proteins encoded by PCR products). In designing such primers, it is advisable to retain at least 15 to 18 contiguous nucleotides on the gene-specific 3′ end of the primer to help ensure correct amplification. The primer design parameters outlined in Table 1 must be strictly adhered to within this gene-specific region to maintain primer specificity. However, it is also essential to verify that the desired adaptor sequence does not have significant similarity to sequences elsewhere in the target DNA.

Primer modifications can also be introduced to permit site-directed mutagenesis at either single or multiple residues. One of the most commonly used methods utilizes two vector-specific primers in conjunction with a single mutagenic primer.[4] In this method, two PCR reactions are carried out. The first PCR contains one vector-specific primer and the mutagenic primer. The second PCR contains the second vector-specific primer and the whole of the first reaction as the second primer.

Several important considerations must be taken into account in designing mutagenic primers for this procedure. The mismatched nucleotide(s) must be located toward the center of the primer. Ideally the mutations would be located toward the 5′ end of the primer so that the 3′ end is highly specific and therefore anneals correctly during the first PCR. However as the product of the first PCR becomes the primer for the second PCR, the 3′ end of the antisense strand must also be allowed to anneal correctly. Hence in practice, mismatches should not be incorporated too close to the 5′ end of the original mutagenic primer. When multiple mismatches are to be introduced into a single primer, the primer T_m and specificity will be reduced. To compensate for this reduction, extra specific residues must be added. In general, it is advisable to add 1.5 bases to a primer per mismatch. Successful mutagenic primers range from between 20 and 24 nucleotides long, containing single mismatches, up to 36 nucleotides long, with eight mismatches.

Another parameter affecting
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IV. COMPU

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Programs for primer design
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Another parameter affecting the choice of primer for this mutagenic approach is that Taq polymerase often catalyzes the non-template directed addition of a residue (often but not always adenine) to the 3′ end of the strand.[13] As the synthesized strands from the first PCR are used directly as a primer in the second PCR, incorrect bases are frequently inserted into the product. To circumvent this, special consideration must be given to the location of the 5′ end of the primer. By siting the 5′ end immediately adjacent to a T residue, the deleterious effect of the addition of an adenine to the opposite strand can be nullified.[15] However, as residues other than adenine are often added and sequences around the location of the desired mutation may often lack appropriately positioned T residues, a more commonly applicable rule is to site the 5′ end of the primer adjacent to a nucleotide in the wobble position of a codon.[16] This will often result in no change to the protein product. By combining these two strategies, mutagenic primers can be designed that direct only the desired changes to protein coding sequences. Adherence to the above rules is also necessary for other PCR methods involving multiple primers and several PCR reactions to resynthesize DNA fragments.[17,18]

IV. COMPUTER-AIDED PCR PRIMER DESIGN

PCR primer design relies on the accurate assessment of a plethora of standard parameters (Table 1). A series of largely empirical considerations can then be considered to design primers for specific applications or product modifications (Table 2). Many of the latter category of parameters can be determined manually. However, it is extremely laborious and time consuming to optimize all the basic parameters for selecting primer pairs. Several computer programs are available to facilitate this analysis.[19,20] These programs search for primers based on optimum sizes and accurate T_ms calculated using the nearest-neighbor method.[5] Additional searches are done to eliminate primers that have non-standard base composition and are capable of forming hairpins. Special attention is given to ensuring the "uniqueness" of the primer sequence within the target DNA, with care given to ensure stable and specific annealing of the 3′ end of the primer to the DNA. Once suitable primers have been selected, "matching pairs" are selected that have compatible T_ms, are spaced correctly, and are not likely to form primer-dimers.

The program *OLIGO* (National BioSciences) is a purpose-designed package for either PC-based or Macintosh computers and can be used for general oligonucleotide design as well as for the selection of PCR primer pairs. Both versions are user friendly and perform all the basic tasks in primer design (Table 1). In addition, this program calculates the annealing temperature of PCR reactions, taking into account the stability of the PCR product[8,20] as well as the primer T_m values. Optimization of this parameter can increase both the specificity and yield of product. The PC version of *OLIGO* also has the additional feature of being able to back-translate protein sequences. This can facilitate the design of degenerate oligonucleotides used in cloning (Table 2).

Programs for primer design are also available from Intelligenetics but are incorporated into more extensive DNA analysis packages. These programs are also available in two versions, *PCGene* for PC-based and *GeneWorks* for Macintosh computers. Again, both versions are user friendly and able to adequately perform the basic steps in primer design (Table 1). In common with the *OLIGO* program, these programs are able to calculate the optimum annealing temperature of the PCR reaction and use it in the selection of primer pairs. Both *PCGene* and *GeneWorks* allow the determination of the uniqueness of the primer pairs in the whole of the *GenBank* database. This task is obviously beyond manual methods, and for many applications it is an essential parameter to consider in primer design. In addition, *PCGene* has the advantage of allowing a primer to be inserted manually and subsequently analyzed, and then a compatible primer can be selected. This function is of considerable importance when primers that contain modifications are to be used in the PCR (Table 2).

V. CONCLUSIONS

Careful oligonucleotide primer design is an essential step in obtaining a successful PCR amplification. A plethora of parameters have been identified that must be optimized to improve the chances of success in a PCR reaction. Particular care must be taken to ensure the correct annealing of the 3′ end of the primer. This is a task that can often be accomplished manually. However, it is obviously impractical to consider all the necessary parameters using manual methods alone. Nevertheless many manually designed primer pairs function well whereas some primer pairs that appear to fulfill all known criteria fail to produce a PCR product. This emphasizes that we have yet to learn all there is to know about rules governing PCR primer design. As more rules are developed, computer programs will become an absolute necessity to cope with this analysis. Indeed, the use of a computer program in designing primers is already strongly advised to simplify this process. One should not, however, lose sight of the fact that the more elaborate primer manipulations are still best done with manual methods due to their empirical nature. I therefore strongly recommend the use of computer assistance, allied to some manual adjustments, many of which can be done within the programs themselves. This combined approach should pay dividends in ensuring consistently successful PCR amplifications.

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